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Selection and Validation of a Multilocus Variable-Number Tandem-Repeat Analysis Panel for Typing *Shigella* spp.▽†

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The *Shigella* genus has historically been separated into four species, based on biochemical assays. The classification within each species relies on serotyping. Recently, genome sequencing and DNA assays, in particular the multilocus sequence typing (MLST) approach, greatly improved the current knowledge of the origin and phylogenetic evolution of *Shigella* spp. The *Shigella* and *Escherichia* genera are now considered to belong to a unique genomospecies. Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) provides valuable polymorphic markers for genotyping and performing phylogenetic analyses of highly homogeneous bacterial pathogens. Here, we assess the capability of MLVA for *Shigella* typing. Thirty-two potentially polymorphic VNTRs were selected by analyzing in silico five *Shigella* genomic sequences and subsequently evaluated. Eventually, a panel of 15 VNTRs was selected (i.e., MLVA15 analysis). MLVA15 analysis of 78 strains or genome sequences of *Shigella* spp. and 11 strains or genome sequences of *Escherichia coli* distinguished 83 genotypes. *Shigella* population cluster analysis gave consistent results compared to MLST. MLVA15 analysis showed capabilities for *E. coli* typing, providing classification among pathogenic and nonpathogenic *E. coli* strains included in the study. The resulting data can be queried on our genotyping webpage (http://mlva.u-psud.fr). The MLVA15 assay is rapid, highly discriminatory, and reproducible for *Shigella* and *Escherichia* strains, suggesting that it could significantly contribute to epidemiological trace-back analysis of *Shigella* infections and pathogenic *Escherichia* outbreaks. Typing was performed on strains obtained mostly from collections. Further studies should include strains of much more diverse origins, including all pathogenic *E. coli* types.

Shigellosis is a widespread disease occurring mainly in developing countries, often in association with poor sanitation, and is responsible for about 600,000 deaths per year in the world, two-thirds of them concerning <10-year-old children (2). The implementation of treatment and, to some extent, control strategies is a significant challenge, especially in Asia, because antibiotic-resistant strains of different species and serotypes have emerged and because the distribution of *Shigella* species and serotypes is heterogeneous (46). The low infectious dose (10 to 100 cells) allows the organism to spread effectively by contaminated food or water but also by person-to-person contact, and a specific virulence plasmid that encodes a type III secretion system plus the invasion plasmid antigens is responsible for epithelial cell wall invasiveness. Finally, it is worth noting that *Shigella*, like other bacteria responsible for food and waterborne diseases, has been classified as a potential biological threat agent due to its infection route and environmental stability (38).

Shigella sp. strains are currently classified into four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. Three of them are apparently antigenically heterogeneous, comprising several serotypes, whereas *S. sonnei* is antigenically homogeneous.

Shigella typing relies on phenotypic characteristics, but discrimination between the four species can be difficult. Serotyping is not always able to provide a correct species identification, due to cross-reactions or the absence of agglutination. New serotypes are regularly discovered (28, 49) and are sometimes found to cross-react with *Escherichia coli* strains.

In addition, the discriminatory power of phenotypic tools and serotyping is limited and requires the manipulation of the live agent. The introduction of DNA-based molecular typing methods, such as ribotyping (5), plasmid profile analysis, restriction fragment length polymorphism (25, 26), and pulsed-field gel electrophoresis (PFGE) (37), has greatly improved the ability of researchers to discriminate between epidemiologically related and unrelated isolates in outbreaks. In the United States, PulseNet, a network of laboratories implicated in foodborne disease surveillance (42), uses PFGE typing coupled with strict quality control procedures in order to ensure interlaboratory reproducibility, but this approach remains labor-intensive for routine clinical strain typing, so cheaper alternatives are actively being pursued. Multilocus sequence typing (MLST) is a very powerful approach, and it provides a clear view of the population structure (52). However, it is not yet appropriate for the routine, first-line genotyping of a large number of isolates. Recently, PulseNet members acknowledged that multilocus variable-number tandem-repeat (VNTR) analysis

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TABLE 1. List of strains used in this study

Genotype	Genus and species	Serotype or serovar	Collection	Alias	Origin[a]	Cluster	Yr of isolation	Location of isolation	Strain identifier
1	*Shigella dysenteriae*	5	CIP 56.17		CRBIP	1	1956	Vietnam	Sd#1
2	*Shigella dysenteriae*	5	CIP 57.42		CRBIP	1	1957	Tunisia	Sd#2
3	*Shigella boydii*	3	CIP 52.50	NCTC 9329	CRBIP	1	1952	Egypt	Sb#3
4	*Shigella boydii*	6	CIP 52.53	NCTC 9332	CRBIP	1	1952		Sb#4
5	*Shigella boydii*	10	CIP 73.2		CRBIP	1	1973		Sb#5
6	*Shigella boydii*	1	NCTC 9327		CRBIP	1	1938		Sb#6
7	*Shigella boydii*	1	CIP 54.73		CRBIP	1	1954	Burkina Faso	Sb#7
8	*Shigella boydii*	8	CIP 51.3		CRBIP	1	1951	Congo	Sb#8
9	*Shigella boydii*	18	BS512		Sanger	1		AZ	Sb#9
10	*Shigella dysenteriae*	3	CIP 59.1		CRBIP	1	1959	Cameroon	Sd#10
11	*Shigella dysenteriae*	3	CIP 67.58		CRBIP	1	1967	Iraq	Sd#11
12	*Shigella dysenteriae*	4	CIP 59.2		CRBIP	1	1959	Cameroon	Sd#12
13	*Shigella dysenteriae*	4	CIP 67.59		CRBIP	1	1967	Iraq	Sd#13
14	*Shigella dysenteriae*	3	CIP 54.77		CRBIP	1	1954	Vietnam	Sd#14
15	*Shigella dysenteriae*	11	CIP 58.16	NCTC 9349	CRBIP	1	1958		Sd#15
16	*Shigella dysenteriae*	9	CIP 58.25	NCTC 9348	CRBIP	1	1958		Sd#16
17	*Shigella boydii*	2	CIP 82.50T	NCTC 12985	CRBIP	1	1982		Sb#17
18	*Shigella boydii*	4	CIP 54.76		CRBIP	1	1954	Vietnam	Sb#18
19	*Shigella boydii*	4	227		ChMPH	1	1950	China	Sb#19
20	*Shigella boydii*	14	CIP 58.17	NCTC 9766	CRBIP	1	1958		Sb#20
21	*Shigella boydii*	14	CIP 58.18	NCTC 9765	CRBIP	1	1958		Sb#21
22	*Shigella flexneri*	6	CIP 106202		CRBIP	1	1940	United Kingdom	Sf#22
23	*Shigella boydii*	14	CIP 53.44	NCTC 6721	CRBIP	1	1953		Sb#23
24	*Shigella flexneri*	6	CIP 55.19		CRBIP	1	1955	Vietnam	Sf#24
25	*Shigella dysenteriae*	6	CIP 52.32		CRBIP	1	1952		Sd#25
26	*Shigella dysenteriae*	7	CIP 52.123	NCTC 9763	CRBIP	1	1952		Sd#26
27	*Shigella dysenteriae*	7	CIP 67.60		CRBIP	1	1967	Iraq	Sd#27
28	*Shigella boydii*	9	CIP 57.47	NCTC 9356	CRBIP	2	1957		Sb#28a
28	*Shigella boydii*	9	CIP 51.4	NCTC 9355	CRBIP	2	1951		Sb#28b
29	*Shigella boydii*	16	CIP 67.11		CRBIP	2	1967	Hong Kong	Sb#29
30	*Shigella boydii*	5	CIP 56.36		CRBIP	2	1956	Ethiopia	Sb#30
31	*Shigella boydii*	11	CIP 51.6		CRBIP	2	1951		Sb#31
32	*Shigella boydii*	11	CIP 56.18		CRBIP	2	1956	Vietnam	Sb#32
33	*Shigella dysenteriae*	2	CIP 54.51		CRBIP	2	1954	Burkina Faso	Sb#33
34	*Shigella dysenteriae*	2	CIP 54.57		CRBIP	2	1954	Vietnam	Sd#34
35	*Shigella boydii*	15	CIP 58.19	NCTC 9365	CRBIP	2			Sb#35
36	*Shigella boydii*	15	CIP 58.20	NCTC 10024	CRBIP	2	1958		Sb#36
37	*Shigella boydii*	7	CIP 58.4		CRBIP	2	1958	Tunisia	Sb#37
38	*Shigella boydii*	7	CIP 52.54	NCTC 333	CRBIP	2	1952		Sb#38
39	*Shigella sonnei*	None	CIP 55.56		CRBIP		1955	Sri Lanka	Ss#39a
39	*Shigella sonnei*	None	CIP 63.10		CRBIP		1963		Ss#39b
40	*Shigella sonnei*	None	53G		Sanger			Japan	Ss#40
41	*Shigella sonnei*	None	CIP 106345		CRBIP		1999	France	Ss#41
42	*Shigella sonnei*	None	CIP 51.44		CRBIP		1951		Ss#42
43	*Shigella sonnei*	None	CIP 104223	ATCC 25931	CRBIP		1994	Panama	Ss#43
44	*Shigella sonnei*	None	46		ChMPH		1950	China	Ss#44
45	*Shigella sonnei*	None			Bégin				Ss#45
46	*Shigella sonnei*	None			Bégin				Ss#46
47	*Escherichia coli*				Bégin		2000	France	Ec#47
48	*Escherichia coli*		CFT073		Univ. of Wisconsin		1990	MD	Ec#48
49	*Escherichia coli*		UTI89		Univ. Goettingen				Ec#49
50	*Escherichia coli*		536		Washington Univ.				Ec#50
51	*Shigella flexneri*	1	CIP 107169		CRBIP	3	1995	France	Sf#51
52	*Shigella flexneri*	2	CIP 106236		CRBIP	3			Sf#52
53	*Shigella flexneri*	4b	CIP 52.43	NCTC 8336	CRBIP	3	1947	United Kingdom	Sf#53
54	*Shigella flexneri*	Y	CIP 52.36		CRBIP	3	1952		Sf#54
55	*Shigella flexneri*	1	CIP 106171		CRBIP	3			Sf#55
56	*Shigella boydii*	12	CIP 58.23	NCTC 9772	CRBIP	3	1958		Sb#56
57	*Shigella flexneri*	Untyped	CIP 54.58		CRBIP		1954	Vietnam	Sf#57
58	*Shigella flexneri*	Untyped	CIP 106210		CRBIP				Sf#58
59	*Shigella flexneri*	2a	301		ChMPH	3	1984	China	Sf#59
60	*Shigella flexneri*	2a	CIP 56.19		CRBIP	3	1956	Tunisia	Sf#60
61	*Shigella flexneri*	2a	ATCC 700930		Univ. of Wisconsin	3		Japan	Sf#61
62	*Shigella flexneri*	2b	CIP 52.39		CRBIP	3	1952		Sf#62

Continued on following page

TABLE 1—*Continued*

Genotype	Genus and species	Serotype or serovar	Collection	Alias	Origin[a]	Cluster	Yr of isolation	Location of isolation	Strain identifier
63	*Shigella flexneri*	5	CIP 67.61		CRBIP	3	1967	Iraq	Sf#63
64	*Shigella flexneri*	5	8401		ChMPH	3			Sf#64
65	*Shigella flexneri*	3	CIP 54.40		CRBIP	3	1919	United Kingdom	Sf#65
66	*Shigella flexneri*	X	CIP 52.34		CRBIP	3	1952		Sf#66
67	*Shigella flexneri*	3a	CIP 106211		CRBIP	3			Sf#67
68	*Shigella dysenteriae*	8	CIP 53.134	NCTC 9345	CRBIP		1953		Sd#68
69	*Shigella flexneri*	4c	CIP 52.25		CRBIP	3	1952		Sf#69
70	*Shigella dysenteriae*	1	CIP 56.33		CRBIP		1956	Ethiopia	Sd#70
71	*Shigella dysenteriae*	1	CIP 106200		CRBIP		1919		Sd#71
72	*Shigella dysenteriae*	1	A147		Bégin		1994	Rwanda	Sd#72
73	*Shigella dysenteriae*	1	A1-313		Bégin		1994	Rwanda	Sd#73
74	*Shigella dysenteriae*	1	197		ChMPH		1950	China	Sd#74a
74	*Shigella dysenteriae*	1	M131649		Sanger		1970	Guatemala	Sd#74b
75	*Shigella dysenteriae*	1	CIP 58.1		CRBIP		1958	Tunisia	Sd#75a
75	*Shigella dysenteriae*	1	CIP 62.17		CRBIP		1962	Cameroon	Sd#75b
76	*Escherichia coli*	O157:H7	Sakaï		Osaka Univ.		1996	Japan	Ec#76
77	*Escherichia coli*	O157:H7	EDL933		Univ. of Wisconsin		1982	MI	Ec#77
78	*Escherichia coli*		K12		CEB		1922	CA	Ec#78a
78	*Escherichia coli*		K12		CEB		1922	CA	Ec#78b
78	*Escherichia coli*		MG1655		Univ. of Wisconsin		1922	CA	Ec#78c
79	*Escherichia coli*		HB101		CEB		1922	CA	Ec#79
80	*Escherichia coli*		W3110		NARA IST		1922	CA	Ec#80
81	*Shigella boydii*	13	CIP 58.21	NCTC 9361	CRBIP		1958		Sb#81
82	*Shigella boydii*	13	CIP 58.22	NCTC 9362	CRBIP		1958		Sb#82
83	*Salmonella enterica*	Typhimurium	LT2		Washington Univ.		1940s		Se#83

[a] Bégin, Hôpital d'Instruction des Armées, Bégin, France; ChMPH, Microbial Genome Center of the Chinese Ministry of Health; CRBIP, Centre de Ressources Biologiques de l'Institut Pasteur; NARA IST, Nara Institute of Science and Technology; Univ., University.

(MLVA) is a highly promising typing tool, likely to replace PFGE in the coming years (9). MLVA typing is being actively developed by a number of laboratories, together with the associated Internet-based query tools and databases (3, 6, 17, 18), to genotype several bacterial pathogens, in particular, potential biothreat agents, such as *Bacillus anthracis* (18), *Yersinia pestis* (18, 31), *Legionella pneumophila* (32, 33), *Salmonella enterica* (36), *Brucella* spp. (19), *Francisella tularensis* (11), *Burkholderia mallei*, and *B. pseudomallei* (43). MLVA is also increasingly recognized as a future reference technique for bacterial genotyping allowing systematic typing of all isolates for a number of other pathogens of high public health interest (including *Mycobacterium tuberculosis* [17], *Pseudomonas aeruginosa* [30, 47], *Streptococcus pneumoniae* [13], and *Staphylococcus aureus* [40]) (the technique is reviewed in references 21, 44, and 45).

The present work aimed to set up MLVA for the *Shigella* genus to facilitate epidemiological follow-up, by providing easy-to-use typing tools, in countries suffering from a recrudescence of *Shigella* outbreaks. Recently, Liang et al. (20) proposed an MLVA for molecular typing of *S. sonnei* and explored the capability of a 26-VNTR set for detecting clusters of infection. Because the proposed VNTRs have very short repeat units, this assay requires a high precision of DNA length measurement, as provided, for instance, by microcapillary electrophoresis and fluorescent markers. This is a strong limitation for routine typing because of the cost of such equipment and the associated consumables. In contrast, the present investigation favors markers with larger repeat units in order to provide an assay widely accessible to research and public health laboratories, particularly in developing countries.

MATERIALS AND METHODS

Strains and DNA preparation. Sixty-eight *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* strains and four *E. coli* strains were obtained from the Centre de Ressources Biologiques de l'Institut Pasteur (CRBIP), the American Type Culture Collection, the Hôpital d'Instruction des Armées (Bégin, France), and the Centre d'Etudes du Bouchet (CEB) strain collections (Table 1). Strains were cultured overnight at 30°C on Trypticase soy agar (reference number 43011; bioMérieux, Marcy L'Etoile, France) before DNA extraction. *S. flexneri*, *S. sonnei*, and *S. boydii* DNA extractions were processed in a biosafety level 2 lab by use of lysozyme, sodium dodecyl sulfate, and proteinase K followed by phenol-chloroform extraction and ethanol precipitation, as described elsewhere (41). *S. dysenteriae* serotype 1 DNA extraction was done in a biosafety level 3 lab by use of the genomic DNA 500/G extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. In several cases, the presence of high quantities of polysaccharides was suspected after lysis and cetyltrimethylammonium bromide extraction was used to remove this excess (41). The average size of the extracted DNA was checked on a 0.8% agarose gel. Nucleic acid concentration and purity were quantified with an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

MLVA setup. A multiple comparison of *Shigella* genome sequences available at the onset of the study was done through the Microorganism Tandem Repeats database (3, 6), accessible as a Web service (http://minisatellites.u-psud.fr/). The methods previously described (3, 18) and the genome sequence data for strains *S. flexneri* 2457T (GenBank accession no. AE005174) (48), *S. flexneri* 301 (GenBank accession no. NC_004337) (10), *S. sonnei* 46 (GenBank accession no. NC_007384), *S. dysenteriae* 197 (GenBank accession no. CP000034), and *S. boydii* 227 (GenBank accession no. NC_007613) (50) were used to identify presumably polymorphic tandem repeats (TRs).

Loci with repeat units longer than 9 bp were favored in order to facilitate allele repeat number calling by a variety of DNA amplicon length estimation devices (24) and as a complement to a previous investigation (20). Selected primers pairs were validated in silico with available genome sequence data for *Shigella* and *E.*

coli strains by using the multiple-PCR-primer BLAST Web service (http://minisatellites.u-psud.fr/) which provides the expected size and sequence of the PCR products. The resulting in silico typing data were integrated into the MLVA.

Some VNTR loci of *E. coli* O157:H7 described by Keys et al. (12) are present in the *Shigella* genus genome sequences. Five of them (O157-11, O157-13, O157-25, O157-33, and O157-34), selected for their relative ease of use (repeat unit size, >6 bp) or high rate of polymorphism, were evaluated in this study.

VNTR amplification and genotyping. PCRs were performed as previously described (18) and using an annealing temperature of 60°C. DNA from the *S. flexneri* 2457T strain was used as an internal control to ensure high-quality size assignments as described previously (36). The PCR products were run on agarose gels, stained with ethidium bromide, visualized under UV, and photographed as previously described (19).

Sequence-based typing. To allow comparison between MLVA and MLST assays, a region of the *thrC* gene described by Pupo et al. that carries enough information to discriminate the collection in the clusters previously described (35) was selected. Primers were designed with Primer3 software (39) and led to a 351-bp-long amplicon. This region was amplified in all strains of the present collection, and PCR products were sent to MWG-BIOTECH AG (Ebersberg, Germany) for sequencing.

Data analysis. Gel electrophoresis images were analyzed by using the Bionumerics software package, version 5.0 (Applied-Maths, Sint-Martens-Latem, Belgium), as previously described (19). The number of repeats in each allele was deduced from the amplicon size. The resulting data were analyzed as a character data set with Bionumerics software. Clustering analysis was done by using the categorical parameter and the unweighted-pair group method with arithmetic averages (UPGMA) coefficient. The same weight was given to large and small numbers of differences in the repeats at each locus. The categorical parameter was also used to calculate the minimum spanning tree (MST). MST is a convenient complementary tool to cluster multiple isolates and visualize the relative diversity within different lineages. Polymorphism was quantified by the Hunter-Gaston diversity index (HGDI) (7). MLST sequences published by Pupo et al. (35) were imported into the Bionumerics program.

RESULTS

The in silico multiple-genome comparison of five genomes identified 32 TRs (repeat unit size, >9 bp; repeat sequence conservation, >80%) predicted to show at least two alleles among the five available genomes. One of the 32 loci was previously investigated (22). Five VNTR loci described by Keys et al. (12) for MLVA typing of *E. coli* O157:H7 were added to this panel owing to their presence and their polymorphism in both *Shigella* spp. and *E. coli* O157:H7. All VNTR loci considered herein are located on the chromosome. Specific primers for these VNTRs were checked using an initial selection of 12 bacterial strains (1 *S. dysenteriae* strain, 4 *S. flexneri* strains, 2 *S. sonnei* strains, 4 *S. boydii* strains, and 1 *E. coli* strain) and led to the elimination of 18 markers (8 failed to amplify DNA satisfactorily and 10 did not show polymorphism) (see Table S1 in the supplemental material). Analysis of the collection of 72 *Shigella* strains (Table 1) eliminated four additional markers that amplified less than 90% of the strains (see Table S1 in the supplemental material), leading to a final set of 15 markers (Table 2), including 3 from the panel described by Keys et al.

The sizes of the amplification products for the sequenced strain *S. flexneri* 2457T were as expected for each marker, and DNA from this strain was used as the reference in all analyses (Fig. 1). Four VNTR loci yielded an allele assignation for all *Shigella* and *Escherichia* strains investigated, while 11 others yielded no PCR product in some strains in spite of repeated attempts, suggesting locus absence and/or sequence polymorphism at priming sites.

Table 3 highlights characteristics and diversity of the 15 selected VNTR loci. Ten loci are located within coding or putative coding regions, and five are intergenic. Repeat unit lengths ranged from 6 to 375 base pairs. These VNTRs were also tested in silico by using the 17 available sets of genomic sequence data of *Shigella*, *E. coli*, and *Salmonella enterica* serovar Typhimurium (Table 1).

The HGDI (7) of each VNTR was calculated for the complete collection and for each species. Three different panels of VNTR loci (panel 1 included 11 loci, panel 2 included 3 loci, and panel 3 included 1 locus) were defined according to their HGDI value and were subsequently combined in a composite data set using three different levels of weight (individual marker weight of 20, 10, or 1) along the line previously proposed for *Brucella* MLVA (1). The rationale for this approach is that the diversity index indirectly reflects mutation rate and homoplasy level at each locus. Markers with a higher homoplasy level have a lower phylogenetic value. Panel 1 included 11 VNTRs (individual weights of 20) with HGDIs below 0.75 (ms09, ms11, ms18, ms22, ms23, ms24, ms25, ms26, ms32, O157-13, and O157-33), panel 2 included 3 VNTRs (individual weights of 10) with HGDIs ranging from 0.75 to 0.9 (ms06, CNV-001, and ms21), and panel 3 is limited to the hypervariable VNTR, O157-11 (individual weight of 1), with an HGDI higher than 0.9.

Diversity indexes differ within the genus *Shigella* and the species *E. coli* (Table 3). As expected, the least variable group of organisms is the *S. sonnei* genus, with an HGDI of 0.64 when the highly variable locus O157-11 is not included (MLVA14, comprising panels 1 and 2). Locus O157-11 alone has an HGDI of 0.94 for *S. sonnei* and leads to a general HGDI of 0.97 for this species. *S. flexneri*, *S. boydii*, and *S. dysenteriae* show much greater MLVA14 diversity, correlated with the higher number of serotypes (12, 15, and 10, respectively) in these species. Regarding *E. coli*, the limited number of strains included in the study does not allow comparison with the others.

By use of the MLVA14 assay (panels 1 and 2), the 89 strains (72 DNA samples tested and 17 sets of sequence data) were differentiated into 65 genotypes. When the O157-11 VNTR is used, the discriminatory power of the assay is significantly increased, with 83 genotypes numbered 1 to 83 in the dendrogram produced (Fig. 2). With the low relative weight given in the clustering analysis to this very highly variable marker, the two clusters are highly similar. All strains are identified by species acronym and genotype number (e.g., Sd#01 or Sf#70). Several strains sharing the same genotype will be additionally differentiated by a letter (e.g., a, b, etc.).

Cluster analysis shows that most of the *Shigella* strains fall into four main clusters. Sb#81 and Sb#82 stand as outliers, Se#83 being an outgroup. Sb#81 and Sb#82 belong to the *S. boydii* serotype 13 (B13 in reference 35) and have an MLVA15 profile very different from those of all the other *Shigella* strains, with seven cases lacking amplification and two alleles observed only in these strains. Two strains of *S. sonnei*, two strains of *S. boydii*, three strains of *E. coli*, and two pairs of *S. dysenteriae* strains share the same genotype. Additional markers might allow further differentiation of *Shigella* strains, supposed to have been isolated in different places at different times (20). The HGDIs, however, are high and, by adding in the cluster analysis markers discarded previously, the strains remain un-

TABLE 2. List of TRs selected

Locus[a]	TR	*S. flexneri* 2457T chromosome location (positions)	Primer sequence		Product	Reference
			Upper	Lower		
flex0880_39bp_301bp_2U	ms06	880507–880807	AAACGGGAGAGCCGGTTATT	TGTTGGTACAACGGCTCCTG	Cell division protein	This report
flex0881_39bp_626bp_7U	CVN001 (ms07)	881365–881990	GTCAGTTCGCCCAGACACAG	CGGTGTCAGCAAATCCAGAG	Cell division protein	22
flex1282_179bp_535bp_2U	ms09	1282058–1282592	GTGCCATCGGGCAAAATTAG	CCGATAAGGGAGCAGGCTAGT		This report
flex1724_96bp_766bp_5U	ms11	1724780–1725545	GAAACAGGCCCAGGCTACAC	CTGGCGCTGGTTATGGGTAT	Electron transport	This report
flex2172_23bp_123bp_2U	ms18	2172858–2172980	AACAAGAATATGAAAAATCA TAGCAC	TTGTAAATTCGGTCATGATAATA ATTC	complex protein RnfC	This report
flex2786_141bp_646bp_4U	ms21	2786095–2786740	GCTGATGGCGAAGGAG AAGA	GGGAGTATGCGGTCAAAAGC		This report
flex2917_9bp_117bp_7U	ms22	2917680–2917796	TGGATACCCGATAACGCAGT	CCTGAAGAATCAACAAGGCTTTA	Prepilin peptidase-dependent protein C	This report
flex3171_375bp_1334bp_3U	ms23	3171829–3173162	GCTCCGCTGATTGACTCCTT	CGGTTGCTCGACCACTAACA		This report
flex3237_78bp_316bp_2U	ms24	3237723–3238038	CGTTTAGCCGAGGATGAAGC	TGTGCGTTAGCAAGCCATTC	Hypothetical protein	This report
flex3406_17bp_340bp_3U	ms25	3406473–3406812	CCGTACAAGCTGCTGATGCT	TGCGTACATCGTCTCGAACA	Hypothetical protein	This report
flex3450_18bp_134bp_2U	ms26	3450707–3450840	TTCTTCTCCAGGTCTACAAA AATC	GCAACAAAACGACATCTTTACCTG		This report
flex4573_101bp_456bp_2U	ms32	4573218–4573673	TGAGATTGCCGAAGTGTTGC	AACTGGCGGCGTTTATCAAG	Hypothetical protein	This report
flex3996_6bp_220bp_6U	O157-11	3776930–3777149	GATGCTGGAAAAACTGATGC AGACTCGCGT	GACCGGCAATCATCGGGCCAACCA	Uroporphyrinogen III methylase	12
flex3659_9bp_121bp_2U	O157-13	4114040–4114160	GCAGCAAACGCCACAGTACC CATGCC	GTAGGTCATCTGCCGTGGTTCGAG CGCT	Hypothetical protein	12
flex4448_16bp_176bp_1U	O157-33	4440283–4440458	GTGAAGGATAAGCTGCATTT GTCAGTGATGTCCGAAG	GCCTGACGCTAAAGATAAAGAAG AAAGCGTCGCG	Hypothetical protein	12

[a] Locus names were created as follows: four letters for the species strain used as a reference, four digits for genome location in the sequenced reference strain, TR size, locus size in the reference strain, and number of TR in the reference strain, separated by underscore sign.

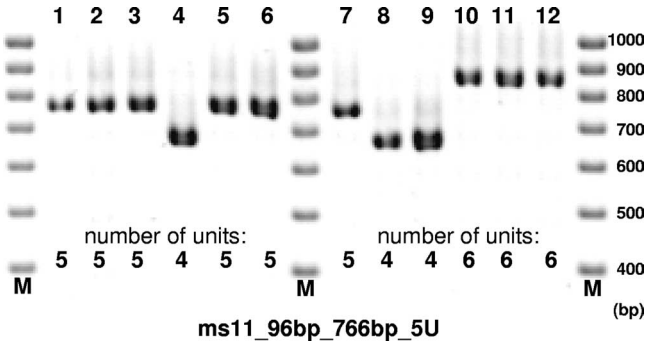


FIG. 1. Illustration of the MLVA assay setup. The PCR products of an amplification using ms06 primers were loaded on an agarose gel, electrophoresed, and stained with ethidium bromide. Lanes M show a 100-bp-ladder molecular weight marker. Lanes 1 and 7 correspond to the *S. flexneri* strain 2457T; lanes 2 to 6 and lanes 8 to 12 correspond to strains from our assay. The image illustrates how the number of units can be directly deduced by manual reading. The marker name below the gel provides the repeat unit size of the TR, the expected PCR product size in the *S. flexneri* 2457T genome, and the corresponding number of units in the *S. flexneri* 2457T genome.

distinguishable (data not shown), supporting the idea that they are in all likelihood epidemiologically related.

To estimate the validity of the clustering observed by VNTR typing, selected strains were analyzed by sequence typing using published MLST data (35). Strains provided amplification products of the expected size, regarding the segment of the *thrC* gene. Sequences were imported and aligned in the Bionumerics program, and a cluster analysis was produced (see Table S2 in the supplemental material). A single discrepancy between clusters inferred from known genera and serotypes on one hand and sequence analysis on the other hand was observed due to the incongruence of phylogeny at the different loci. *S. dysenteriae* CIP 53.134 (D8 in reference 35) fell within cluster 3 when only the *thrC* sequence data were analyzed. It is the *trpC-trpB* sequence used in the MLST assay, in particular, which excludes that strain from cluster 3. Subsequently, all strains from the collection for which the *thrC* locus was investigated were characterized by their relevance to the clusters classified by Pupo et al., as indicated in Fig. 2.

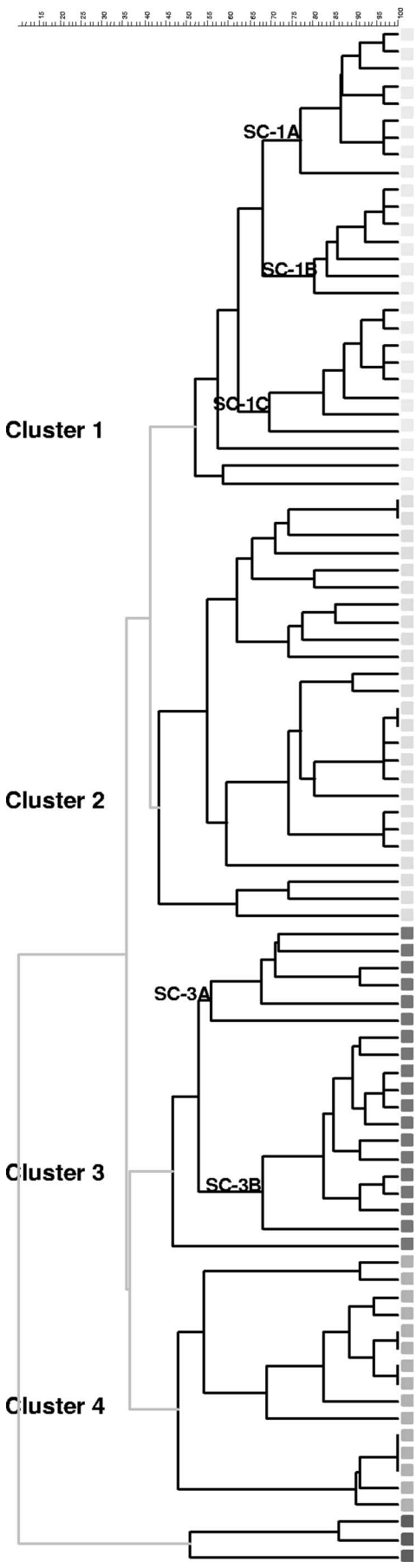
This sequence analysis and comparison with published sequence data show that three of these clusters do correspond to the clusters classified by Pupo et al., and they are numbered accordingly in Fig. 2. Cluster 1 grouped *S. boydii* serotypes 1 to 4, 6, 8, 10, 14, and 18, *S. dysenteriae* serotypes 3 to 7, 9, and 11, and *S. flexneri* serotype 6. Cluster 1 was the most diverse, grouping representatives of all *Shigella* species except *S. sonnei*. This cluster could be divided into three subclusters, i.e., SC-1A, SC-1B, and SC-1C. Strains sharing the same serotype are usually discriminated by MLVA15 typing.

Cluster 2 comprises *S. boydii* serotypes 5, 7, 9, 11, 15, and 16, *S. dysenteriae* serotype 2, and all *S. sonnei* serotypes. The two *S. boydii* serotype 9 displayed the same MLVA15 pattern. The *S. sonnei* strains are located into two branches, one with the two representatives of *S. boydii* serotype 7. Three uropathogenic *E. coli* strains are included in cluster 2, together with an *E. coli*

TABLE 3. Diversity indexes calculated for MLVA panels and individual markers in the four *Shigella* species and *E. coli*[a]

Locus	Alias	Total (n = 89)		*E. coli* (n = 11)		*S. boydii* (n = 26)		*S. dysenteriae* (n = 23)		*S. flexneri* (n = 19)		*S. sonnei* (n = 9)	
		No. of genotypes	HGDI	No. of genotypes	HGDI	No. of genotypes	HGDI	No. of genotypes	HGDI	No. of genotypes	HGDI	No. of genotypes	HGDI
ms09		8	0.65	4	0.75	5	0.69	3	0.53	3	0.4	2	0.22
ms11		5	0.51	3	0.59	2	0.41	4	0.74	2	0.22	1	0.00
ms18		4	0.27	1	0.00	2	0.14	2	0.47	3	0.23	1	0.00
ms22		6	0.53	2	0.33	3	0.22	2	0.36	5	0.67	1	0.00
ms23		5	0.40	3	0.56	2	0.21	4	0.25	2	0.51	1	0.00
ms24		4	0.35	2	0.18	3	0.22	1	0.00	2	0.38	1	0.00
ms25		6	0.38	1	0.00	3	0.22	2	0.09	4	0.71	1	0.00
ms26		5	0.68	2	0.51	4	0.54	4	0.64	2	0.38	2	0.50
ms32		6	0.56	3	0.69	4	0.65	3	0.32	1	0	1	0.00
O157-13		5	0.42	2	0.33	4	0.65	3	0.37	2	0.12	1	0.00
O157-33		4	0.50	2	0.33	1	0.00	2	0.47	1	1	2	0.50
Panel 1		53	0.98	8	0.89	14	0.95	14	0.93	15	0.96	3	0.64
ms06		6	0.77	2	0.51	6	0.77	6	0.79	2	0.52	1	0.00
CNV-001	ms07	11	0.78	4	0.75	7	0.70	4	0.66	4	0.42	1	0.00
ms21		7	0.76	4	0.75	3	0.69	5	0.68	3	0.59	2	0.56
Panels 1 and 2		65	0.99	9	0.95	19	0.97	18	0.98	16	0.98	3	0.64
O157-11 (panel 3)		19	0.93	5	0.85	15	0.96	6	0.81	10	0.92	7	0.94
Total for MLVA-15		83	1.00	9	0.95	25	1.00	21	0.99	19	1.00	8	0.97

[a] n, number of strains.



genotype	genus	species	serotype	collection	Pupo's clustering	isolation date	geographic origine
1	Shigella	dysenteriae	5	CIP 56.17	1	1956	Vietnam
2	Shigella	dysenteriae	5	CIP 57.42	1	1957	Tunisia
3	Shigella	boydii	3	CIP 52.50	1	1952	Egypt
4	Shigella	boydii	6	CIP 52.53	1	1952	.
5	Shigella	boydii	10	CIP 73.2	1	1973	.
6	Shigella	boydii	1	NCTC 9327	1	1938	.
7	Shigella	boydii	1	CIP 54.73	1	1954	Burkina-Faso
8	Shigella	boydii	8	CIP 51.3	1	1951	Congo
9	Shigella	boydii	18	BS512	1		USA (AZ)
10	Shigella	dysenteriae	3	CIP 59.1	1	1959	Cameroon
11	Shigella	dysenteriae	3	CIP 67.58	1	1967	Iraq
12	Shigella	dysenteriae	4	CIP 59.2	1	1959	Cameroon
13	Shigella	dysenteriae	4	CIP 67.59	1	1967	Iraq
14	Shigella	dysenteriae	3	CIP 54.77	1	1954	Vietnam
15	Shigella	dysenteriae	11	CIP 58.16	1	1958	.
16	Shigella	dysenteriae	9	CIP 58.25	1	1958	.
17	Shigella	boydii	2	CIP 82.50T	1	1982	.
18	Shigella	boydii	4	CIP 54.76	1	1954	Vietnam
19	Shigella	boydii	4	227	1	1950	China
20	Shigella	boydii	14	CIP 58.17	1	1958	.
21	Shigella	boydii	14	CIP 58.18	1	1958	.
22	Shigella	flexneri	6	CIP 106202	1	1940	UK
23	Shigella	boydii	14	CIP 53.44	1	1953	.
24	Shigella	flexneri	6	CIP 55.19	1	1955	Vietnam
25	Shigella	dysenteriae	6	CIP 52.32	1	1952	.
26	Shigella	dysenteriae	7	CIP 52.123	1	1952	.
27	Shigella	dysenteriae	7	CIP 67.60	1	1967	Iraq
28	Shigella	boydii	9	CIP 57.47	2	1957	.
28	Shigella	boydii	9	CIP 51.4	2	1951	.
29	Shigella	boydii	16	CIP 67.11	2	1967	Hong-Kong
30	Shigella	boydii	5	CIP 56.36	2	1956	Ethiopia
31	Shigella	boydii	11	CIP 51.6	2	1951	.
32	Shigella	boydii	11	CIP 56.18	2	1956	Vietnam
33	Shigella	dysenteriae	2	CIP 54.51	2	1954	Burkina-Faso
34	Shigella	dysenteriae	2	CIP 54.57	2	1954	Vietnam
35	Shigella	boydii	15	CIP 58.19	2		
36	Shigella	boydii	15	CIP 58.20	2	1958	.
37	Shigella	boydii	7	CIP 58.4	2	1958	Tunisia
38	Shigella	boydii	7	CIP 52.54	2	1952	.
39	Shigella	sonnei	none	CIP 55.56	2	1955	Sri Lanka
39	Shigella	sonnei	none	CIP 63.10	2	1963	.
40	Shigella	sonnei	none	53G	2		Japan
41	Shigella	sonnei	none	CIP 106345	2	1999	France
42	Shigella	sonnei	none	CIP 51.44	2	1951	.
43	Shigella	sonnei	none	CIP 104223	2	1994	Panama
44	Shigella	sonnei	none	046	2	1950	China
45	Shigella	sonnei	none		2		.
46	Shigella	sonnei	none		2		.
47	Escherichia	coli			.	2000	France
48	Escherichia	coli		CFT073	.	1990	USA (MD)
49	Escherichia	coli		UTI89	.		
50	Escherichia	coli		536	.		
51	Shigella	flexneri	5	CIP 67.61	3	1967	Iraq
52	Shigella	flexneri	5	8401	3		
53	Shigella	flexneri	3	CIP 54.40	3	1919	UK
54	Shigella	flexneri	X	CIP 52.34	3	1952	.
55	Shigella	flexneri	3a	CIP 106211	3		.
56	Shigella	dysenteriae	8	CIP 53.134	.	1953	.
57	Shigella	flexneri	1	CIP 107169	3	1995	France
58	Shigella	flexneri	2	CIP 106236	3		.
59	Shigella	flexneri	4b	CIP 52.43	3	1947	UK
60	Shigella	flexneri	Y	CIP 52.36	3	1952	.
61	Shigella	flexneri	1	CIP 106171	3		
62	Shigella	boydii	12	CIP 58.23	3	1958	.
63	Shigella	flexneri	untyped	CIP 54.58	3	1954	Vietnam
64	Shigella	flexneri	untyped	CIP 106210	3		.
65	Shigella	flexneri	2a	301	3	1984	China
66	Shigella	flexneri	2a	CIP 56.19	3	1956	Tunisia
67	Shigella	flexneri	2a	ATCC 700930	3		Japan
68	Shigella	flexneri	2b	CIP 52.39	3	1952	.
69	Shigella	flexneri	4c	CIP 52.25	3	1952	.
70	Escherichia	coli	O157:H7	Sakai	4	1996	Japan
71	Escherichia	coli	O157:H7	EDL933	4	1982	USA (MI)
72	Shigella	dysenteriae	1	CIP 56.33	4	1956	Ethiopia
73	Shigella	dysenteriae	1	CIP 106200	4	1919	.
74	Shigella	dysenteriae	1	A147	4	1994	Rwanda
74	Shigella	dysenteriae	1	A1-313	4	1994	Rwanda
75	Shigella	dysenteriae	1	197	4	1950	China
75	Shigella	dysenteriae	1	M131649	4	1970	Guatemala
76	Shigella	dysenteriae	1	CIP 58.1	4	1958	Tunisia
77	Shigella	dysenteriae	1	CIP 82.17	4	1962	Cameroon
78	Escherichia	coli		K12	4	1922	USA (CA)
78	Escherichia	coli		MG1655	4	1922	USA (CA)
78	Escherichia	coli		K12	4	1922	USA (CA)
79	Escherichia	coli		HB101	4	1922	USA (CA)
80	Escherichia	coli		W3110	4	1922	USA (CA)
81	Shigella	boydii	13	CIP 58.21	.	1958	.
82	Shigella	boydii	13	CIP 58.22	.	1958	.
83	Salmonella	enterica	liphymurium	LT2	.	1940s	

strain isolated from a patient at Hôpital d'Instruction des Armées, Bégin, France (Ec#47).

Cluster 3 groups all *S. flexneri* strains (with the exception of *S. flexneri* serotype 6, located in cluster 1), *S. boydii* serotype 12, and *S. dysenteriae* serotype 8 (Sd#56). Sd#56 is weakly associated with subcluster 3A together with *S. flexneri* serotypes 3, 3a, 5, and X. A second subcluster, tentatively called 3B (Fig. 2), comprises *S. boydii* serotype 12 and *S. flexneri* serotypes 1, 2, 2a, 2b, 4b, and Y. *S. flexneri* serotype 4c is more distantly related. Each strain has a unique genotype, although several serotypes were represented by more than one strain.

Consequently, and with the exception of Sd#56 (Sd8), the composition of the clusters is in remarkable agreement with the report of Pupo et al., although the underlying approaches are quite different (35).

We propose here to define a fourth cluster. Cluster 4 would include all the *S. dysenteriae* serotype 1 strains, five representatives of the *E. coli* K-12 strain (including two genomic sequences), and the two *E. coli* O157:H7 strains included in the study. The *E. coli* O157:H7 representatives are located in a quite distinct branch, slightly closer to the *S. dysenteriae* serotype 1 branch than to the *E. coli* K-12 branch. The eight *S. dysenteriae* serotype 1 strains are separated into six genotypes, showing the discriminative power of the VNTR panel. Among those strains, two were isolated from patients during an outbreak in a refugee camp in Rwanda in 1994. They show exactly the same MLVA15 pattern. Three of the *E. coli* K-12 strains were wild-type K-12 and two were derivatives (14). The three K-12 wild types share exactly the same genotype, while derivatives present different patterns at locus ms22, ms32, or O157-11. The O157:H7 strains exhibited two differences among them, at loci ms11 and O157-11.

In very rare instances, a given allele is strongly associated with a specific cluster, at least in the limited collection of strains investigated here. The three-repeat-unit allele at locus ms06 is observed only in cluster 2, and locus O157-33 has more than one repeat unit only in clusters 2 and 4 (associated with the *E. coli* strains investigated here).

ms25 and ms26 each gave rise to a very large amplicon in two strains and four strains, respectively, and these amplicons correspond to alleles with more than 60 repeat units (compared to the usual allele size ranges of one to three and two to four repeat units, respectively) (see Table S2 in the supplemental material). The six alleles were sequenced. The ms25 sequencing revealed that two different insertion sequences (ISs) were present, IS*629* and IS*2* for Sf#22 and Sd#26, respectively. IS*629* is a member of the IS*3* family of transposable elements. The ms26 allele sequencing led to the finding that only one IS, known as IS*630*, was present in this TR (27), and it was inserted at the same position and orientation in the four strains Sd#33, Sd#34, Sb#35, and Sb#36, confirming the close relationship between the four strains already suggested by MLVA15 clustering, in spite of a different species assignment.

As a complementary analysis, an MST analysis was performed. MST analysis is a convenient tool to cluster multiple isolates and illustrate the relative diversity within different lineages (Fig. 3). This kind of analysis is applicable to categorical data sets. The creation of hypothetical types further minimizes the summed distance of all branches of the tree. The MST was drawn without locus O157-11, which is too variable to address correct relatedness within different lineages, especially since this analysis does not currently allow the assignment of different relative weights to markers. In cluster 1, the three main subclusters are conserved, with only minor changes. The two *S. dysenteriae* serotype 7 strains, Sd#26 and Sd#27, are located in the vicinity of subcluster A but interspaced by Sb#04 and Sb#05. The composition of cluster 2, with *S. sonnei* and *S. boydii* serotype 7 in the same branch, is also found by this approach. Sd#56 is not included in cluster 3, which is in accordance with results obtained from other molecular methods and reflects the absence of amplification at several loci, which is taken into account in this analysis. The only change in cluster 4 is the location of *E. coli* O157:H7 representatives that are far from other members of the cluster and weakly linked to cluster 2.

DISCUSSION

The *Shigella* genus has been classified into species and serotypes for a long time and for practical reasons, but MLST (15, 16, 34, 52) and genome comparisons (51) have emphasized the strong genetic homogeneity between the different species. Most importantly, MLST has further strongly suggested that in some instances, the current species designations are not compatible with the actual phylogeny of the *Shigella* genus. It is now considered more likely that *Shigella* sp. strains, as well as enteroinvasive *E. coli* strains, are derived from multiple origins within the species *E. coli* (50).

The purpose of the present study was to develop an MLVA assay for *Shigella* and evaluate if the resulting data would be in agreement with the complex concept of the *Shigella-E. coli* genomospecies, as revealed by MLST data. We investigated TR loci predicted to be polymorphic by comparing five *Shigella* genome sequences. The selection of 15 VNTRs allowed us to discriminate 89 strains, covering the large majority of known diversity in *Shigella* species and several *E. coli* strains (77 *Shigella* spp., 11 *E. coli* strains, and 1 *S. enterica* strain), into 83 genotypes. Strains sharing the same serotype are rarely from the same genotype, with difference ranging from one marker (Sd#1 and Sd#2, of *S. dysenteriae* serotype 5) to five markers (Sd#26 and Sd#27, of *S. dysenteriae* serotype 7). On the other hand, the two strains of *S. dysenteriae* serotype 1 isolated in Rwanda in 1994 during a dysentery outbreak in refugee camps are identical.

Through MLVA15 analysis, four main clusters were defined, together with some outliers. Among them, the strains of *S.*

FIG. 2. Dendrogram with all the typed strains, including the ones corresponding to sequenced strains that were included in the data set by their allele numbers as calculated theoretically from the expected amplicon sizes of all 15 loci based on their genome sequences. Clustering analysis was done using the categorical and UPGMA options. The columns indicate the genotype number, the genus, the species, the serotype, the strain identification number (collection), the cluster number as defined by Pupo et al., and the year and place of isolation.

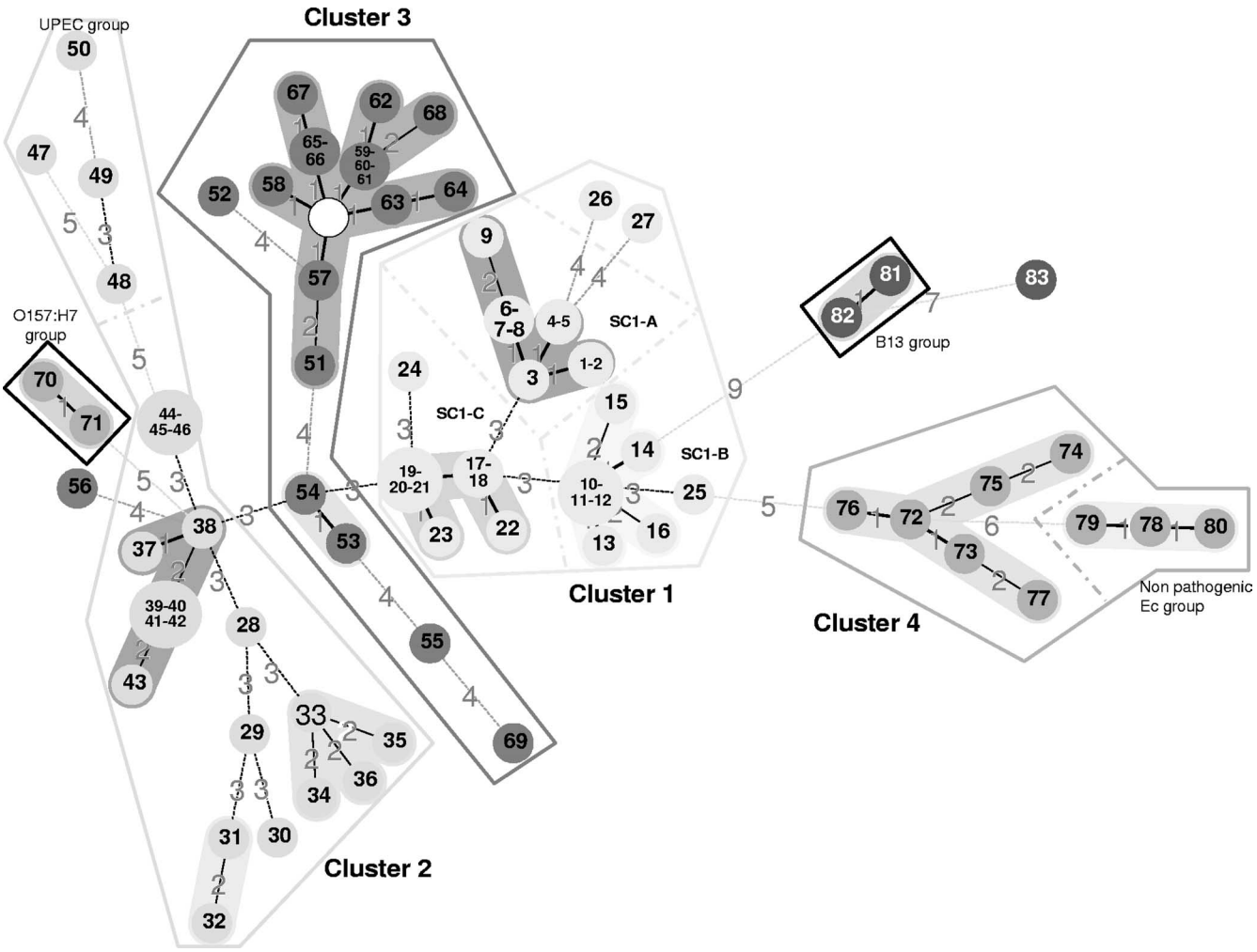


FIG. 3. *Shigella* population modeling. The number in each circle indicates the corresponding genotype identified in Fig. 2. The empty circle indicates a hypothetical genotype (not present in the population analyzed) created to minimize overall distances between neighboring genotypes. The distance between neighboring genotypes is expressed as the number of allelic changes and is outlined by different shapes of lines: a hatched bold line indicates one change, a full gray line indicates two changes, a black dotted line indicates three changes, and a gray dotted line indicates more than four changes. Gray hatched lines are used to separate subgroups inside clusters. The tree calculation was made without locus O157-11, owing the location of different genotypes in the same circle.

boydii serotype 13 were clearly distinguished from the other *Shigella* strains. Some cases lacking amplification, as well as unique sizes for several loci, illustrate the wide distance between *S. boydii* serotype 13 and other *Shigella* representatives. According to Hyma et al. (8), *S. boydii* serotype 13 is closely related to a different *Escherichia* species, *E. albertii*. It supports the hypothesis of an ancient separation of lineages, *S. boydii* serotype 13, as well as *S. enterica* LT2, being a clear outgroup. This finding also corroborated the results of MLST investigations by Pupo et al. (35) and Lan et al. (15).

In order to be able to compare our MLVA15 clustering data with published MLST data, part of the *thrC* gene was sequenced. In this way, the strains used in this study could be assigned to the different clusters defined by Pupo et al. (35), and it very clearly appears that MLVA15 clustering provides the same view of the *Shigella* population structure as MLST. Recently, Yang et al. (52) conducted another MLST analysis, based on 23 housekeeping genes. The greater number of genes analyzed allowed them to define three subclusters inside cluster 1. MLVA15 clustering reveals the same fine substructure. Such striking similarity demonstrates the potential usefulness of MLVA for *Shigella* typing. While the general patterns are highly similar, there are some differences between MLST and MLVA approaches. In the present study, the nine *S. sonnei* strains are assigned to cluster 2, close to *S. boydii* serotype 7. Yang et al. (52) placed the single *S. sonnei* investigated close to cluster 1 by MLST. The *S. sonnei* strain investigated by Escobar-Paramo et al. (4) was found to be closer to cluster 2 and cluster 3 by analysis of four other housekeeping genes. Pupo et al. (35) placed the single *S. sonnei* strain they analyzed far from the three clusters. Thus, the definitive location of *S. sonnei* strains remains undefined. Further MLST analysis of *S. sonnei* might eventually permit us to revisit the relative evolutionary position of this species.

Considering that *Shigella* clusters are now believed to have independently arisen several times from *E. coli* species

while acquiring pathogenicity factors (16, 35, 52), the underrepresentation of *E. coli* investigated here led to the representation of small groups of *E. coli* strains arising from *Shigella* clusters, while, in fact, the situation would be the opposite. Such analysis should be done by including a *Shigella* collection in a larger *E. coli* collection to obtain a general overview of *E. coli*/*Shigella* sp. genomospecies (such work is in progress).

Combining UPGMA cluster analysis and MST provides an overview of *Shigella* intraspecies relationships. In agreement with previous investigations, we can infer from this combination that *Shigella* taxonomy is of little phylogenetic value. This is illustrated for instance by *S. flexneri* serotype 6 being associated with cluster 1 by MLVA or MLST, whereas the other *S. flexneri* serotypes are assigned to cluster 3 (together with the two serologically untyped *S. flexneri* strains). Four strains clustered together by MLVA share the same rare IS insertion event at the same position, while they are assigned to different species, reinforcing the fact that current classification does not reflect the genetic relatedness within the *Shigella* genus. It is now believed that *Shigella* organisms arose several times within *E. coli* species (35, 52), leading to three clusters not related to the currently recognized *Shigella* species taxonomy based upon biochemical and serological characteristics. The three main clusters are also identified here with a fourth corresponding essentially to *S. dysenteriae* serotype 1.

This study augments the results relative to discrimination power, specificity, and sensitivity of the MLVA approach to the *Shigella* species. Moreover, the MLVA15 panel shows some capabilities to be applied to *E. coli* for typing purposes, with a wider range of use than considered before in several published studies, mainly focused on Shiga toxin-producing *E. coli* (12, 23, 29). Another MLVA assay was validated for O157:H7 outbreak detection (12). It showed high discrimination between the *E. coli* O157 strains and appeared to have equal sensitivity to that of PFGE and specificity superior to that of PFGE. Lindstedt et al. (22) described a seven-TR panel designed to type the ECOR collection and pathogenic *E. coli* species. Several *Shigella* strains were included in the study and typed. However, all serotypes were not available, and in one case, the TR was absent, and in another, the TR was monomorphic. Our panel primarily intended to cover all *Shigella* species, and its satisfactory performance indicates also good capabilities to discriminate between nonpathogenic *E. coli*, uropathogenic *E. coli*, and enterohemorrhagic *E. coli* strains.

As suggested previously (45), the proposed combination of well-selected independent polymorphic TR loci is highly discriminatory and provides a relevant clustering compared with the currently accepted classification. The panel of 15 markers has been divided into three panels according to the polymorphism of each locus. It is important to keep in mind that the very high discriminatory power of some markers usually results in a very high homoplasy level. For this reason, such markers must be given a lower weight when similarity matrices are calculated. Here, the different weights proposed for each panel were empirically determined, but it is very likely that in future MLVA developments, special attention will be given to the optimization of these coefficients. For this to be done, much larger collections of strains, with detailed epidemiological data, will need to be typed first. It can only be hoped that in the future, funding bodies will support international consortiums of laboratories aimed at producing high-quality data of this kind. VNTR typing could then be widely accessible for research and public health laboratories, particularly in developing countries, where the majority of cases of *Shigella* occur, since this method is highly suitable for sharing results and for the generation of databases as previously demonstrated (http://mlva.u-psud.fr).

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REFERENCES

1. **Al Dahouk, S., P. Le Flèche, K. Nockler, I. Jacques, M. Grayon, H. C. Scholz, H. Tomaso, G. Vergnaud, and H. Neubauer.** 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J. Microbiol. Methods **69:**137–145.
2. **Chaignat, C.** 2004. Shigellosis, p. 527–531. *In* D. L. Heyman (ed.), Control of communicable diseases manual, 18th ed. American Public Health Association, Washington, DC.
3. **Denoeud, F., and G. Vergnaud.** 2004. Identification of polymorphic tandem repeats by direct comparison of genome sequence from different bacterial strains: a web-based resource. BMC Bioinformatics **5:**4.
4. **Escobar-Paramo, P., C. Giudicelli, C. Parsot, and E. Denamur.** 2003. The evolutionary history of *Shigella* and enteroinvasive *Escherichia coli* revised. J. Mol. Evol. **57:**140–148.
5. **Faruque, S. M., K. Haider, M. M. Rahman, A. R. Abdul Alim, Q. S. Ahmad, M. J. Albert, and R. B. Sack.** 1992. Differentiation of *Shigella flexneri* strains by rRNA gene restriction patterns. J. Clin. Microbiol. **30:**2996–2999.
6. **Grissa, I., P. Bouchon, C. Pourcel, and G. Vergnaud.** On-line resources for bacterial micro-evolution studies using MLVA or CRISPR typing. Biochimie, in press.
7. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
8. **Hyma, K. E., D. W. Lacher, A. M. Nelson, A. C. Bumbaugh, J. M. Janda, N. A. Strockbine, V. B. Young, and T. S. Whittam.** 2005. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. J. Bacteriol. **187:**619–628.
9. **Hyytia-Trees, E., S. C. Smole, P. A. Fields, B. Swaminathan, and E. M. Ribot.** 2006. Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis of Shiga toxin-producing Escherichia coli O157 (STEC O157). Foodborne Pathog. Dis. **3:**118–131.
10. **Jin, Q., Z. Yuan, J. Xu, Y. Wang, Y. Shen, W. Lu, J. Wang, H. Liu, J. Yang, F. Yang, X. Zhang, J. Zhang, G. Yang, H. Wu, D. Qu, J. Dong, L. Sun, Y. Xue, A. Zhao, Y. Gao, J. Zhu, B. Kan, K. Ding, S. Chen, H. Cheng, Z. Yao, B. He, R. Chen, D. Ma, B. Qiang, Y. Wen, Y. Hou, and J. Yu.** 2002. Genome sequence of *Shigella flexneri* 2a: insights into pathogenicity through comparison with genomes of *Escherichia coli* K12 and O157. Nucleic Acids Res. **30:**4432–4441.
11. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol. **186:**5808–5818.
12. **Keys, C., S. Kemper, and P. Keim.** 2005. Highly diverse variable number tandem repeat loci in the *E. coli* O157:H7 and O55:H7 genomes for high-resolution molecular typing. J. Appl. Microbiol. **98:**928–940.
13. **Koeck, J. L., B. M. Njanpop-Lafourcade, S. Cade, E. Varon, L. Sangare, S. Valjevac, G. Vergnaud, and C. Pourcel.** 2005. Evaluation and selection of tandem repeat loci for *Streptococcus pneumoniae* MLVA strain typing. BMC Microbiol. **5:**66.
14. **Kuhnert, P., J. Nicolet, and J. Frey.** 1995. Rapid and accurate identification of *Escherichia coli* K-12 strains. Appl. Environ. Microbiol. **61:**4135–4139.
15. **Lan, R., M. C. Alles, K. Donohoe, M. B. Martinez, and P. R. Reeves.** 2004. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. Infect. Immun. **72:**5080–5088.
16. **Lan, R., and P. R. Reeves.** 2002. *Escherichia coli* in disguise: molecular origins of *Shigella*. Microbes Infect. **4:**1125–1132.

17. **Le Flèche, P., M. Fabre, F. Denoeud, J. L. Koeck, and G. Vergnaud.** 2002. High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. BMC Microbiol. **2:**37.
18. **Le Flèche, P., Y. Hauck, L. Onteniente, A. Prieur, F. Denoeud, V. Ramisse, P. Sylvestre, G. Benson, F. Ramisse, and G. Vergnaud.** 2001. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. BMC Microbiol. **1:**2.
19. **Le Flèche, P., I. Jacques, M. Grayon, S. Al Dahouk, P. Bouchon, F. Denoeud, K. Nockler, H. Neubauer, L. A. Guilloteau, and G. Vergnaud.** 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. **6:**9.
20. **Liang, S. Y., H. Watanabe, J. Terajima, C. C. Li, J. C. Liao, S. K. Tung, and C. S. Chiou.** 2007. Multilocus variable-number tandem repeat analysis for molecular typing of *Shigella sonnei*. J. Clin. Microbiol. **45:**3574–3580.
21. **Lindstedt, B. A.** 2005. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis **26:** 2567–2582.
22. **Lindstedt, B. A., L. T. Brandal, L. Aas, T. Vardund, and G. Kapperud.** 2007. Study of polymorphic variable-number of tandem repeats loci in the ECOR collection and in a set of pathogenic Escherichia coli and Shigella isolates for use in a genotyping assay. J. Microbiol. Methods **69:**197–205.
23. **Lindstedt, B. A., E. Heir, E. Gjernes, T. Vardund, and G. Kapperud.** 2003. DNA fingerprinting of Shiga-toxin producing *Escherichia coli* O157 based on multiple-locus variable-number tandem-repeats analysis (MLVA). Ann. Clin. Microbiol. Antimicrob. **2:**12.
24. **Lista, F., G. Faggioni, S. Valjevac, A. Ciammaruconi, J. Vaissaire, C. le Doujet, O. Gorgé, R. De Santis, A. Carattoli, A. Ciervo, A. Fasanella, F. Orsini, R. D'Amelio, C. Pourcel, A. Cassone, and G. Vergnaud.** 2006. Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci multiple locus variable-number tandem repeats analysis. BMC Microbiol. **6:**33.
25. **Litwin, C. M., A. L. Storm, S. Chipowsky, and K. J. Ryan.** 1991. Molecular epidemiology of *Shigella* infections: plasmid profiles, serotype correlation, and restriction endonuclease analysis. J. Clin. Microbiol. **29:**104–108.
26. **Liu, P. Y., Y. J. Lau, B. S. Hu, J. M. Shyr, Z. Y. Shi, W. S. Tsai, Y. H. Lin, and C. Y. Tseng.** 1995. Analysis of clonal relationships among isolates of *Shigella sonnei* by different molecular typing methods. J. Clin. Microbiol. **33:**1779–1783.
27. **Matsutani, S., H. Ohtsubo, Y. Maeda, and E. Ohtsubo.** 1987. Isolation and characterization of IS elements repeated in the bacterial chromosome. J. Mol. Biol. **196:**445–455.
28. **Melito, P. L., D. L. Woodward, J. Munro, J. Walsh, R. Foster, P. Tilley, A. Paccagnella, J. Isaac-Renton, J. Ismail, and L. K. Ng.** 2005. A novel *Shigella dysenteriae* serovar isolated in Canada. J. Clin. Microbiol. **43:**740–744.
29. **Noller, A. C., M. C. McEllistrem, A. G. Pacheco, D. J. Boxrud, and L. H. Harrison.** 2003. Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. J. Clin. Microbiol. **41:**5389–5397.
30. **Onteniente, L., S. Brisse, P. T. Tassios, and G. Vergnaud.** 2003. Evaluation of the polymorphisms associated with tandem repeats for *Pseudomonas aeruginosa* strain typing. J. Clin. Microbiol. **41:**4991–4997.
31. **Pourcel, C., F. Andre-Mazeaud, H. Neubauer, F. Ramisse, and G. Vergnaud.** 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. BMC Microbiol. **4:**22.
32. **Pourcel, C., Y. Vidgop, F. Ramisse, G. Vergnaud, and C. Tram.** 2003. Characterization of a tandem repeat polymorphism in *Legionella pneumophila* and its use for genotyping. J. Clin. Microbiol. **41:**1819–1826.
33. **Pourcel, C., P. Visca, B. Afshar, S. D'Arezzo, G. Vergnaud, and N. K. Fry.** 2007. Identification of variable-number tandem repeat sequences in *Legionella pneumophila* and development of an optimized MLVA typing scheme. J. Clin. Microbiol. **45:**1190–1199.
34. **Pupo, G. M., D. K. Karaolis, R. Lan, and P. R. Reeves.** 1997. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. Infect. Immun. **65:**2685–2692.
35. **Pupo, G. M., R. Lan, and P. R. Reeves.** 2000. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc. Natl. Acad. Sci. USA **97:**10567–10572.
36. **Ramisse, V., P. Houssu, E. Hernandez, F. Denoeud, V. Hilaire, O. Lisanti, F. Ramisse, J. D. Cavallo, and G. Vergnaud.** 2004. Variable number of tandem repeats in *Salmonella enterica* subsp. *enterica* for typing purposes. J. Clin. Microbiol. **42:**5722–5730.
37. **Ribot, E. M., M. A. Fair, R. Gautom, D. N. Cameron, S. B. Hunter, B. Swaminathan, and T. J. Barrett.** 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog. Dis. **3:**59–67.
38. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.
39. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132:**365–386.
40. **Sabat, A., J. Krzyszton-Russjan, W. Strzalka, R. Filipek, K. Kosowska, W. Hryniewicz, J. Travis, and J. Potempa.** 2003. New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J. Clin. Microbiol. **41:**1801–1804.
41. **Sambrook, J., and D. Russell.** 2001. Molecular cloning, a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
42. **Swaminathan, B., T. J. Barrett, S. B. Hunter, and R. V. Tauxe.** 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. **7:**382–389.
43. **U'Ren, J. M., J. M. Schupp, T. Pearson, H. Hornstra, C. L. Friedman, K. L. Smith, R. R. Daugherty, S. D. Rhoton, B. Leadem, S. Georgia, M. Cardon, L. Y. Huynh, D. DeShazer, S. P. Harvey, R. Robison, D. Gal, M. J. Mayo, D. Wagner, B. J. Currie, and P. Keim.** 2007. Tandem repeat regions within the *Burkholderia pseudomallei* genome and their application for high resolution genotyping. BMC Microbiol. **7:**23.
44. **van Belkum, A.** 2007. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS Immunol. Med. Microbiol. **49:**22–27.
45. **Vergnaud, G., and C. Pourcel.** 2006. Multiple locus VNTR (variable number of tandem repeat) analysis (MLVA), p. 83–104. *In* E. Stackebrandt (ed.), Molecular identification, systematics and population structure of prokaryotes, 1st ed. Springer-Verlag, New York, NY.
46. **von Seidlein, L., D. R. Kim, M. Ali, H. Lee, X. Wang, V. D. Thiem, G. Canh do, W. Chaicumpa, M. D. Agtini, A. Hossain, Z. A. Bhutta, C. Mason, O. Sethabutr, K. Talukder, G. B. Nair, J. L. Deen, K. Kotloff, and J. Clemens.** 2006. A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Med. **3:**e353.
47. **Vu-Thien, H., G. Corbineau, K. Hormigos, B. Fauroux, H. Corvol, A. Clement, G. Vergnaud, and C. Pourcel.** 2007. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J. Clin. Microbiol. **45:**3175–3183.
48. **Wei, J., M. B. Goldberg, V. Burland, M. M. Venkatesan, W. Deng, G. Fournier, G. F. Mayhew, G. Plunkett III, D. J. Rose, A. Darling, B. Mau, N. T. Perna, S. M. Payne, L. J. Runyen-Janecky, S. Zhou, D. C. Schwartz, and F. R. Blattner.** 2003. Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. Infect. Immun. **71:** 2775–2786.
49. **Woodward, D. L., C. G. Clark, R. A. Caldeira, R. Ahmed, G. Soule, L. Bryden, H. Tabor, P. Melito, R. Foster, J. Walsh, L. K. Ng, G. B. Malcolm, N. Strockbine, and F. G. Rodgers.** 2005. Identification and characterization of *Shigella boydii* 20 serovar nov., a new and emerging Shigella serotype. J. Med. Microbiol. **54:**741–748.
50. **Yang, F., J. Yang, X. Zhang, L. Chen, Y. Jiang, Y. Yan, X. Tang, J. Wang, Z. Xiong, J. Dong, Y. Xue, Y. Zhu, X. Xu, L. Sun, S. Chen, H. Nie, J. Peng, J. Xu, Y. Wang, Z. Yuan, Y. Wen, Z. Yao, Y. Shen, B. Qiang, Y. Hou, J. Yu, and Q. Jin.** 2005. Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. Nucleic Acids Res. **33:**6445–6458.
51. **Yang, J., L. Chen, J. Yu, L. Sun, and Q. Jin.** 2006. ShiBASE: an integrated database for comparative genomics of *Shigella*. Nucleic Acids Res. **34:**D398–D401.
52. **Yang, J., H. Nie, L. Chen, X. Zhang, F. Yang, X. Xu, Y. Zhu, J. Yu, and Q. Jin.** 2007. Revisiting the molecular evolutionary history of *Shigella* spp. J. Mol. Evol **64:**71–79.